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Enhanced Expression of the Protein Kinase Substrate p36 in Human Hepatocellular Carcinoma

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A basic phosphoprotein defined by a monoclonal antibody named AF5 was found to be highly abundant in human hepatocellular carcinoma by Western immunoblotting. Under the same conditions, the levels of this phosphoprotein were low or undetectable in normal liver extracts. The AF5 antibody was used to screen a cDNA expression library of a human hepatoma cell line named FOCUS. A 960-base-pair cDNA was isolated and found to be a partial cDNA encoding the human protein-tyrosine kinase substrate p36, also known as lipocortin II. p36 expression was highly abundant in hepatocellular carcinomas at both the transcript and protein levels. Its expression was not induced significantly during rat liver regeneration following a partial hepatectomy. These results suggest that the induction of p36 expression is associated with malignant transformation of hepatocytes. p36 was previously shown to be phosphorylated upon transformation of normal fibroblasts by retroviral oncogenes without significant modulation of expression. We report here the initial description of the association of increased p36 expression with malignant transformation.

Several proteins differentially expressed in tumors have been identified by monoclonal antibodies (MAbs) produced against tumor extracts, whole cells, or membrane preparations (for reviews, see references 37 and 38). In order to identify proteins that are present in human hepatocellular carcinoma (HCC) but undetectable in normal liver, we have generated MAbs to a human hepatoma cell line named FOCUS (29, 41). One of these MAbs, named AF5, has allowed us to characterize a basic phosphoprotein antigen. This antigen was not detectable in normal hepatocytes but was present at high levels in primary tumors of hepatocellular origin as well as in HCC-derived cell lines. In this report, we describe the molecular characterization of this phosphoprotein antigen and its identification by cDNA cloning as the protein kinase substrate p36 (21).

MATERIALS AND METHODS

Tissue specimens. HCCs with adjacent nontumorous liver samples were obtained at autopsy or from surgical specimens. Normal adult and fetal human tissues were obtained at autopsy. Peripheral mononuclear leukocytes were obtained from normal volunteers and isolated from whole blood by Ficoll-Hypaque gradient centrifugation. Regenerating rat liver and other tissues were obtained from female Wistar rats. All tissues and cells were stored at −70°C.

Cell lines. The human HCC cell line FOCUS was established in our laboratory (29). Other cell lines employed in these studies are listed in Table 1. These cell lines were obtained from the American Type Culture Collection, Rockville, Md.

Production and characterization of MAb AF5. An early passage of the FOCUS cell line (29) was used to immunize BALB/c mice. The mice first received an intraperitoneal injection (200 μl) of 4×10^6 FOCUS cells in 50% complete Freund adjuvant. Secondary immunizations were performed 6 to 10 weeks later by intravenous inoculation of the same number of living cells suspended in 0.01 M sodium phosphate (pH 7.2) and 0.14 M sodium chloride buffer (phosphate-buffered saline). Three days later, the splenocytes obtained were fused with the parent myeloma cell line SP_2O. Hybrids were selected in medium containing hypoxanthine, aminopterin, and thymidine. Cloning was performed by limiting dilution, and hybridomas were screened for anti-FOCUS antibodies by an indirect binding assay with ^{125}I-labeled sheep F(ab')$_2$ anti-mouse immunoglobulins (specific activity, 8 to 12 μCi/μg of protein; Du Pont, NEN Research Products, Boston, Mass.) as described elsewhere (41). Direct binding assays were performed with protein A affinity-purified MAb AF5 labeled with Na^{125}I (Amersham Corp., Arlington Heights, Ill.) by the Iodo-gen (Pierce Chemical Co., Rockford, Ill.) technique to a specific activity of 12 to 16 μCi/μg of protein as previously described (41). Direct binding assays were carried out in a manner similar to that of the indirect assays except that cells were incubated directly with ^{125}I-AF5 (10^5 cpm in 0.2 ml of phosphate-buffered saline containing 20% calf serum). The filters were counted after a 1-h incubation.

Metabolic studies. Subconfluent FOCUS cells were grown overnight under standard culture conditions and then incubated for 3 h in methionine-free or phosphate-free medium. Cells were labeled with 125 μCi of [^{35}S]methionine (Amersham; specific activity, >1000 Ci/mmol) per ml or with 400 μCi of [^{32}P]orthophosphate (Du Pont, NEN) per ml in 10-cm-diameter culture dishes for 24 h. Cell lysis and immunoprecipitation experiments were performed as described elsewhere (2) except that sodium vanadate was also added in the lysis and washing buffers to a 0.1 mM final concentration. Immunoprecipitation experiments were performed with MAb AF5 and MAb anti-human p36 D1/274 (25) ascitic fluid as well as rabbit antiserum to chick p36 (7) at a 1:200 dilution. The immunoprecipitates were directly suspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (28), heated at 75°C for 5 min, and centrifuged, and the supernatants were subjected to

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TABLE 1. Immunoreactivity of ^{125}I-AF5 MAb with human tumor cell lines and Vero cells as determined by direct binding assay

Cell line	Origin	Binding of AF5 antibody[a]	
		Bound cpm (mean ± SD)	S/N[b]
FOCUS	HCC	2,011 ± 149	56
Hep3B	HCC	2,397 ± 186	67
HepG2	Hepatoblastoma	897 ± 266	25
PLC/PRF/5	HCC	2,993 ± 220	83
SK-HEP-1	Liver adenocarcinoma	2,674 ± 72	74
SK-CO-1	Colon adenocarcinoma	3,302 ± 352	92
SW 403	Colon adenocarcinoma	2,271 ± 196	63
WiDr	Colon adenocarcinoma	3,342 ± 132	93
COLO-320DM	Colon adenocarcinoma	103 ± 20	3
LS 180	Colon adenocarcinoma	2,435 ± 13	68
Caco-2	Colon adenocarcinoma	2,723 ± 340	74
Calu-3	Lung adenocarcinoma	4,285 ± 233	119
Calu-6	Lung anaplastic carcinoma	2,755 ± 421	77
SK-LU-1	Lung adenocarcinoma	2,321 ± 379	64
A-427	Lung carcinoma	2,435 ± 1,084	68
SK-MES-1	Lung squamous carcinoma	1,632 ± 210	45
C-33A	Cervical carcinoma	1,732 ± 154	48
AN3CA	Endometrial adenocarcinoma	1,208 ± 129	34
SK-MEL-5	Malignant melanoma	3,459 ± 400	96
SK-UT-1	Uterine mesodermal leiomyoma	2,900 ± 211	81
Vero	Monkey kidney fibroblasts	2,981 ± 80	83

[a] Each value represents the mean of three measurements with 10^5 cells per assay.

[b] S/N is the ratio obtained by dividing counts per minute (cpm) bound to cell line(s) by counts per minute bound to FOCUS cells preincubated with an excess of unlabeled AF5 antibody. (N = 36 ± 11 cpm.)

SDS-PAGE with 10% polyacrylamide slab gels. After electrophoretic protein separation, the gels were processed for fluorography with Amplify solution (Amersham) according to the instructions of the manufacturer. Autoradiography was carried out at −70°C with X-ray films (Kodak X-Omat AR; Eastman Kodak Co., Rochester, N.Y.).

Partial proteolytic peptide mapping. One-dimensional peptide maps of [^{35}S]methionine-labeled proteins were prepared as described by Cooper and Hunter (7) by digestion with *Staphylococcus aureus* V8 protease during reelectrophoresis through an SDS-polyacrylamide gel (20% polyacrylamide, 0.065% bis-acrylamide).

Western immunoblotting. Detergent-solubilized cell lysates were prepared from confluent cells harvested from culture flasks with a nonproteolytic EDTA buffer. Cells were washed twice in phosphate-buffered saline and resuspended in the lysis buffer (100 mM Tris hydrochloride [pH 8.0], 100 mM NaCl, 0.1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40). After 15 min of incubation on ice with occasional vortexing, the lysates were centrifuged for 15 min at 10,000 rpm at 4°C in an Eppendorf centrifuge 5415C. The supernatants were harvested, adjusted to a protein concentration of 1 mg/ml, and stored frozen at −70°C until use. Detergent-solubilized tissue extracts were prepared similarly by homogenizing tissue samples in 10 volumes of buffer with a Polytron homogenizer. Following homogenization, detergent was added. Cell and tissue extracts adjusted to equal protein concentrations (50 to 100 μg) were separated by SDS-PAGE, electrotransferred to nitrocellulose paper, and analyzed as previously described (33) with the ^{125}I-AF5 antibody.

Indirect immunoperoxidase staining. Hepatocarcinomas and adjacent uninvolved liver samples were obtained at autopsy, immediately frozen in liquid nitrogen, and stored at −80°C. Sections were cut from these snap-frozen tissues and processed for indirect immunostaining as previously described (41). Tissue sections were incubated with the AF5 antibody and stained with the Vectastain ABC kit (Vector Laboratories Inc., Burlingame, Calif.).

Isolation of RNA and preparation of a cDNA library from FOCUS cells. RNA was isolated from FOCUS cells by the guanidinium thiocyanate extraction procedure (30). poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography (31). poly(A)$^+$ RNA (4 μg) was copied into cDNA by reverse transcriptase with oligo(dT) as a primer (27), double-stranded cDNA was prepared, and *Eco*RI linkers were added (19). The cDNA was ligated into a λGT11 expression vector as described previously (24). About 10^6 independent recombinant plaques were obtained. Bacteriophages were plated with *Escherichia coli* Y1088 for amplification before screening (24).

Cloning and characterization of cDNA inserts. The FOCUS cDNA library was screened on blotted filters with ^{125}I-AF5 antibody. Approximately 20,000 PFU were plated with *E. coli* Y1090 (24). After 4 h of incubation at 42°C, the plates were covered with IPTG (isopropyl-β-D-thiogalactopyranoside)-saturated nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) and incubated overnight at 37°C. The blotted filters were briefly washed in 20 mM Tris (pH 7.4) containing 500 mM NaCl (Tris-buffered saline) and blocked by incubating for 30 min in Tris-buffered saline containing 1% nonfat milk (Carnation). Filters were then incubated for 4 h with 0.5×10^6 to 1.0×10^6 cpm of ^{125}I-AF5 antibody per ml in Tris-buffered saline containing nonfat milk at a concentration of 1%. Immunoreactive plaques were identified by autoradiography and subsequently purified. Phage DNA was extracted as described elsewhere (31). cDNA inserts were initially characterized by restriction enzyme analysis, and the longest cDNA insert was sequenced by the dideoxy-chain termination method (32) following subcloning into the PGEM4Z vector (Promega Biotec). Sequence data were analyzed with the GCG sequence analysis software as implemented on a MicroVAX II computer (10).

Northern (RNA) blot analysis. RNA was isolated from various tissues, and 10- to 40-μg samples of each preparation were subjected to electrophoresis under denaturing conditions (31) and transferred onto Nytran membranes (Du Pont, NEN). The RNA was hybridized to randomly primed [^{32}P]cDNA probes (6). All filters were washed at 50 to 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and autoradiographed as described previously (9).

Partial hepatectomy. Two-thirds hepatectomy was performed on eight rats as described previously (40). Two rats were sacrificed at each of the following times: 1, 2, 6, and 24 h following two-thirds hepatectomy. Total RNAs extracted from liver specimens were analyzed as described above.

RESULTS

Production and characterization of MAb AF5. The hybridoma cell line producing MAb AF5 described here was obtained from a cell fusion between SP_2O and BALB/c mouse splenocytes hyperimmunized with FOCUS cells. Following purification and radioiodination of AF5 MAb, the binding of radiolabeled antibody to various hepatoma cell lines was tested. As shown in Table 1, AF5 antibody bound

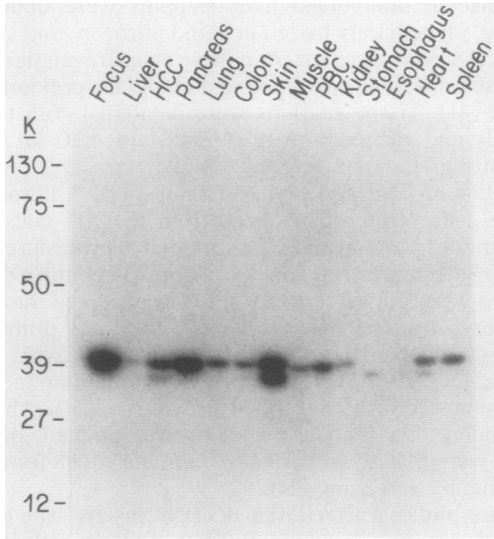

FIG. 1. Immunoblot analysis of the AF5 antigen levels in FOCUS cells and in human adult tissues. Cell and tissue extracts were immunoblotted after SDS-PAGE and detected with ^{125}I-labeled AF5 antibody. PBC, peripheral leukocytes; K, kilodaltons.

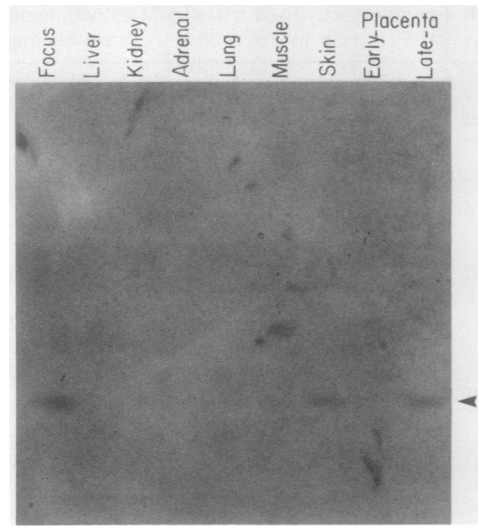

FIG. 2. Immunoblot analysis of the AF5 antigen levels in human fetal tissues (17 weeks old except lane labeled "Placenta Late-" [obtained from a full-term placenta]). Immunoblotting was performed as described in the legend to Fig. 1.

highly to five of five hepatoma cell lines, as shown by signal-to-noise (S/N) ratios between 25 and 83. We also observed high immunoreactivity with 14 other human tumor cell lines tested (S/N ratios, 34 to 119) but not with COLO-320 DM cells of neuroendocrine origin that reacted to ^{125}I-labeled AF5 antibody at a low level. AF5 antibody also bound to the monkey fibroblast cell line Vero very strongly (S/N ratio of 83).

Initial characterization and normal tissue distribution of the AF5 antigen. The antigen recognized by the AF5 antibody was initially characterized by Western immunoblotting. Cell lysates obtained from detergent-extracted FOCUS cells were analyzed by two-dimensional gel electrophoresis followed by Western immunoblotting. We determined by this technique that AF5 antigen was a basic protein. We observed no change in the molecular weight under reducing and nonreducing conditions (data not shown). The expression of AF5 antigen in various human tissues was studied by Western immunoblotting (Fig. 1). We observed a weakly immunoreactive band in detergent-soluble extracts from liver. Other tissues, such as muscle, heart, lung, spleen, kidney, stomach, esophagus, colon, and adrenal gland tissues, showed intermediate levels of immunoreactive antigen. Pancreas and skin tissues expressed very high levels. It is noteworthy that AF5 antigen was undetectable in fetal liver, kidney, adrenal, and muscle tissues but was found to be present in fetal lung and skin tissues. Finally, we demonstrated the presence of AF5 antigen in full-term placentas (Fig. 2).

AF5 antigen in primary hepatocellular carcinomas. An extract obtained from a primary HCC tumor demonstrated high levels of AF5 antigen (Fig. 1); additional HCC tissue extracts were investigated. In Fig. 3, we present a Western immunoblot of HCC and adjacent uninvolved liver pairs derived from eight individuals. We found that AF5 antigen was highly expressed in all tumor specimens but was absent in the adjacent normal liver counterparts, with the notable exception of two specimens. We subsequently found by light microscopy that these two "normal liver specimens" contained metastatic foci of HCC cells. In order to confirm and extend the above observations, we performed immunoperoxidase staining of two pairs of snap-frozen HCC and adjacent normal liver tissues. AF5 antibody demonstrated diffuse cytoplasmic immunostaining of HCC cells (Fig. 4A); adjacent normal hepatocytes did not demonstrate AF5 antigen immunoreactivity (Fig. 4B).

Identification of AF5 antigen as the protein-tyrosine kinase substrate p36. The molecular cloning of a cDNA encoding the AF5 antigen allowed us to establish the identity of this protein. We identified three clones by AF5 immunoreactivity out of 200,000 phages from the λGT11 FOCUS cell library. After digestion with *Eco*RI, we selected the clone showing

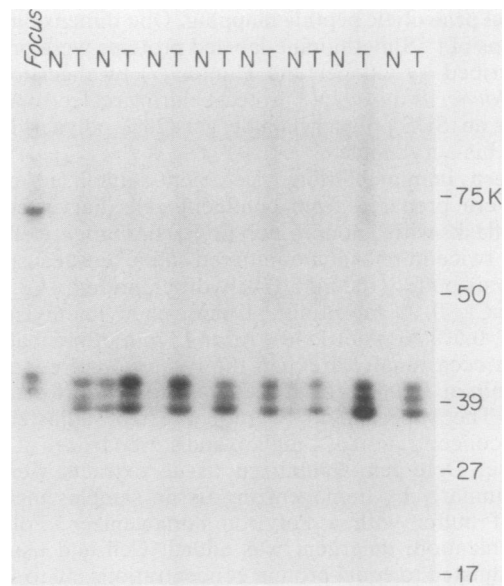

FIG. 3. Immunoblot analysis of AF5 antigen levels in human HCC tumors (T) as compared with adjacent nontumorous liver tissues (N). Immunoblotting was performed as described in the legend to Fig. 1.

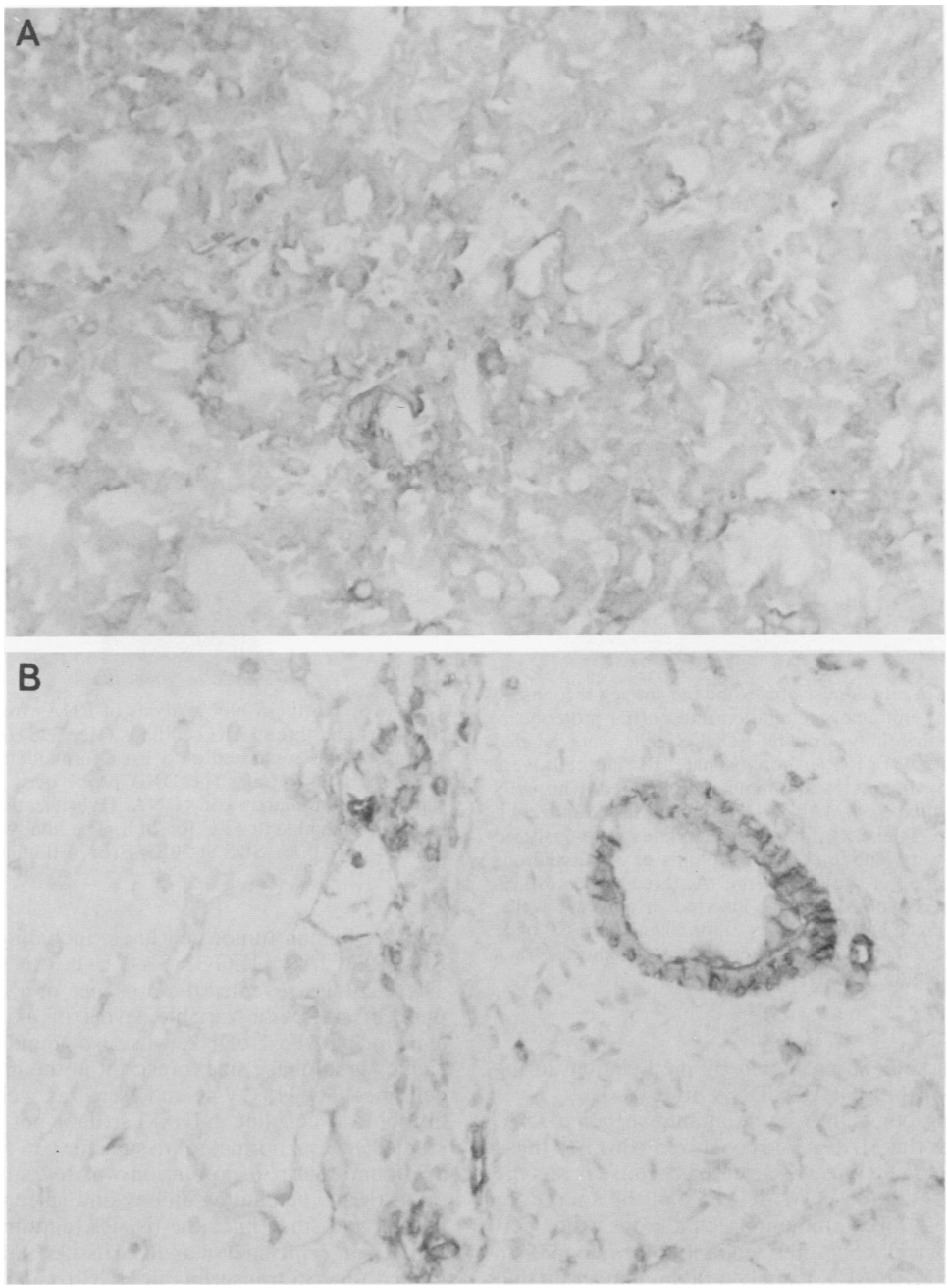

FIG. 4. Indirect immunoperoxidase staining of snap-frozen HCC (A) and adjacent normal liver tissue (B). Tissue sections were immunostained with AF5 antibody as described in Materials and Methods. Note the diffuse cytoplasmic staining and higher-intensity membrane staining seen on most tumor cells (A). In adjacent liver tissue, immunostaining was negative in hepatocytes but positive with Kupffer and biliary epithelial cells (B).

the largest insert (1.0 kilobase pairs) for further investigation. The insert was purified and subcloned into the pGEM4Z plasmid with the unique *Eco*RI site present in the polylinker. The recombinant plasmid was named pGAF5-1 and directly sequenced by the dideoxy-chain termination method. Initially, oligonucleotides corresponding to the T7 and SP6 promoter sequences present on the vector were used as primers. Primers homologous to sequences present in the cDNA insert were used for further sequencing experiments. The pGAF5-1 insert contained a 969-base-pair nucleotide sequence (data not shown). Complete identity was found between residues 8 to 969 of our cDNA and residues 9 to 970 of the published sequence encoding protein-tyrosine kinase substrate p36, also known as lipocortin II (21). The pGAF5-1 cDNA contains an open reading frame encoding 307 amino acids, including the initiator methionine. This putative amino acid sequence was also found to be identical to the p36 sequence between the initiator methionine and the phenylalanine at position 306 near the carboxy-terminal end of the molecule (21). The pGAF5-1 cDNA clone lacks approximately 400 nucleotides spanning the C-terminal coding region as well as the stop codon, the polyadenylation signal, and the poly(A) tail. This is probably due to the presence of an internal *Eco*RI site in the p36 cDNA (21)

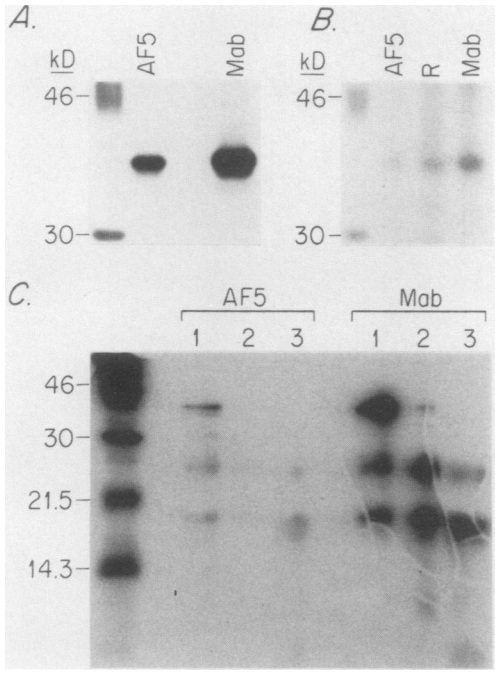

FIG. 5. Analysis of relatedness of p36 and the antigen recognized by the MAb AF5 by immunoprecipitation and partial proteolysis. Two dishes of exponentially growing FOCUS cells were labeled with [^{35}S]methionine (A) or [^{32}P]orthophosphate (B). The cells were lysed, divided into aliquots, and immunoprecipitated with AF5 (lanes AF5), anti-human p36 MAb D1/274 (lanes Mab), and rabbit antiserum to chick p36 (lane R). Immunoprecipitates were analyzed on a 10% SDS-polyacrylamide gel. (C) Proteins of 39 kilodaltons were excised from the gel shown in panel A; three pieces of gels excised from lane AF5 or Mab were inserted in adjacent wells, overlaid with 5 ng (lanes 1), 50 ng (lanes 2), or 500 ng (lanes 3) of *S. aureus* V8 protease, and analyzed by overnight electrophoresis on a 20% SDS-polyacrylamide gel. kD, Kilodaltons.

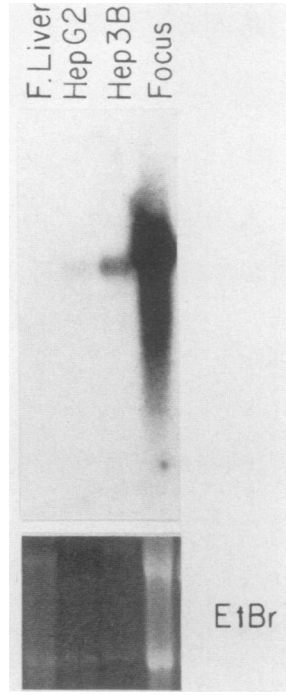

FIG. 6. Northern blot analysis of RNAs from human fetal liver (F.Liver) and three HCC cell lines (HepG2, Hep3B, and FOCUS). The RNA was separated on a 1.0% formaldehyde-agarose gel and transferred to Nytran. The DNA probe used was a 969-base-pair sequence from human p36 cDNA. Hybridizations were carried out in 50% formamide at 42°C for 20 h. The final wash of the filter was in 0.1× SSC–0.1% SDS at 50°C. EtBr, Ethidium bromide.

which may not have been protected by methylation during the construction of the FOCUS cDNA library.

After identifying our cDNA clone as human p36 cDNA, we tested whether the MAb AF5 recognized p36. For these studies, the FOCUS cell line was selected because it was the immunogen for generation of MAb AF5 and the source for our cDNA library. First, immunoprecipitations were performed with two well-characterized antibodies to p36 to compare with the MAb AF5, namely a rabbit antiserum that specifically recognizes p36 in various species, including humans (7, 14), and D1/274, a MAb specific for human p36 (25). Identically migrating 39-kilodalton molecules were immunoprecipitated from [^{35}S]methionine-labeled FOCUS cells by using AF5 and D1/274 MAbs (Fig. 5A). Proteins immunoprecipitated after [^{32}P]phosphate labeling with all three antibodies also migrated identically on an SDS-polyacrylamide gel (Fig. 5B). Finally, the nature of the relationship between p36 and AF5 antigen was corroborated by the patterns generated upon partial proteolytic cleavage of [^{35}S]methionine-labeled proteins immunoprecipitated with the respective monoclonal antibodies (Fig. 5C).

Identification of p36 RNA transcripts. The size of the p36 mRNA derived from different hepatoma cell lines was determined by Northern (RNA) blotting. An RNA band (~1.6 kilobases) was detected in the hepatoma cell lines FOCUS, HepG2, and Hep3B (Fig. 6). We also looked for transcripts in other human tumor cell lines, including COLO-320 DM, SK-BR-3, JAR, JEG-3, SK-LU-1, AN3CA, and C-33A. These studies revealed the presence of a single transcript of uniform size with variable levels of expression (data not shown). pGAF5-1 cDNA was also found to hybridize to a single 1.6-kilobase size transcript in the monkey fibroblastic cell lines COS-1, CV-1, and Vero, as well as in the mouse fibroblastic cell line NIH 3T3 (data not shown). We also studied several normal rat tissues for p36-related transcripts. We found that p36 expression was low in liver and in some other tissues, including kidney and spleen tissues, and high in lung and small intestine tissues (data not shown).

p36 transcripts in human liver tissues and HCC tumors. We looked for p36 transcripts in HCC tissue as compared with their respective adjacent liver counterparts. Figure 7 demonstrates the Northern blots from HCC-normal liver pairs derived from six individuals. High levels of a single 1.6-kilobase transcript were detected in every tumor studied. The normal liver counterparts of the same tumors had undetectable or very low levels of the same transcript. As previously observed, the normal liver counterpart in three patients was found by light microscopy to have tumor metastasis, and thus in these samples we demonstrated the presence of the p36 1.6-kilobase transcript. It was of interest that p36 transcript was not detected in a 17-week-old human fetal liver, as shown in Fig. 6.

p36 expression in regenerating rat liver. In order to clarify whether p36 expression was also associated with proliferation of hepatocytes, we extracted RNA from regenerating rat liver 1, 2, 6, and 24 h after a partial hepatectomy. As shown in Fig. 8, p36 expression was not induced by partial hepa-

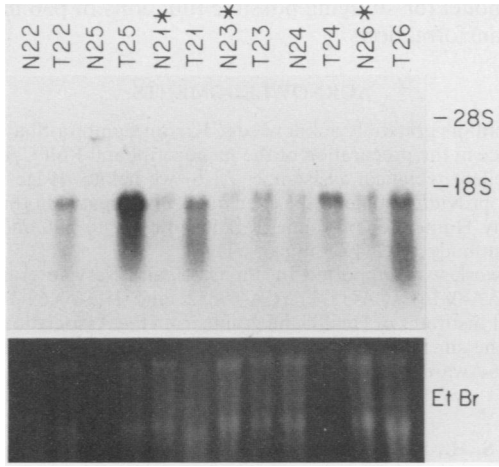

FIG. 7. Northern analysis of RNAs from six pairs of HCC tumor (T) and adjacent nontumorous liver (N) tissues. RNA was analyzed as described in the legend to Fig. 6. "Normal" parts marked with an asterisk (N21, N23, and N26) were shown to be contaminated with metastatic foci of HCC tumors. EtBr, Ethidium bromide.

tectomy in regenerating rat liver tissues at 24 h, when maximal DNA synthesis is observed (40). In contrast, we detected high levels of p36 transcripts in the rat hepatoma cell line FaO (Fig. 8). Liver tissues examined 1, 2, and 6 h after hepatectomy also demonstrated no induction of p36 expression (data not shown). As a control, the same RNA samples were also used to study the induction of ornithine decarboxylase, an enzyme previously shown to be induced during liver regeneration (22). Ornithine decarboxylase transcripts found at low levels in normal liver tissues increased during liver regeneration, with maximum expression reached at 6 h (5- to 10-fold increase) after partial hepatectomy (data not shown; M. Nishiyama et al., submitted for publication).

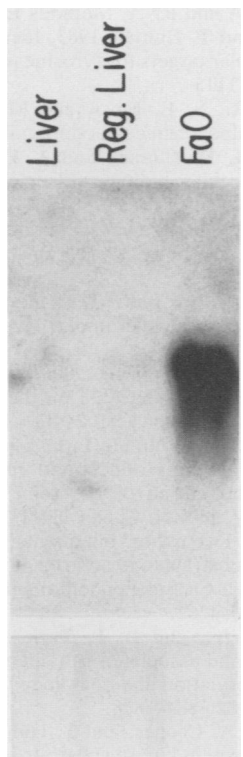

FIG. 8. Northern analysis of RNAs from adult rat livers and rat hepatocarcinoma cell line FaO. RNAs were extracted from liver samples of normal (Liver) or two-thirds-hepatectomized (Reg. Liver, 24 h after hepatectomy) rats as well as from FaO cells. RNAs were analyzed as described in the legend to Fig. 6.

DISCUSSION

Several lines of evidence indicate that the AF5 antigen present in transformed hepatocytes is the previously described protein-tyrosine kinase substrate p36. Structurally, p36 was identified as a basic phosphoprotein of 338 amino acids and a molecular weight of ~38,500 (21, 36). Although it was originally described as a 36-kilodalton protein, further studies demonstrated that it migrated between 34 and 39 kilodaltons on SDS-polyacrylamide gels (15, 23). This apparent heterogeneity of size was thought to be due to variations in experimental conditions as well as the existence of several phosphorylated molecular forms (17, 25). In addition, there may be partially degraded forms of p36 (15). Our studies demonstrated that AF5 antigen was indistinguishable from p36 with respect to phosphorylation, apparent size on SDS-PAGE, and pattern generated upon partial proteolytic cleavage with V8 protease. In addition, the levels of AF5 antigen in different tissues and cell lines were similar to those described for p36. It had been previously shown that p36 was present at the highest levels in skin, lung, and intestine tissues; intermediate levels were noted in spleen, lymph node, thymus, and testes, and low levels were observed in heart and kidney tissues (5, 16, 18). It is noteworthy that p36 was not detected in liver, brain, or skeletal muscle tissues (16, 18, 34). Our findings on the normal tissue distribution of AF5 antigen were similar to the findings of others as mentioned above. As shown in Fig. 1, AF5 immunoreactivity was low in liver tissue. Other tissues, including those from muscle, heart, lung, spleen, kidney, stomach, esophagus, colon, and adrenal glands, showed intermediate levels of p36. In contrast, two normal tissues expressed very high levels (pancreas and skin).

At the cellular level, p36 was found to be differentially expressed in cells derived from different tissues. For example, p36 expression has been found in endothelial cells and fibroblasts of all tissues examined. The expression in other epithelial cells and in muscle cells was variable and dependent in part on the tissues studied (16, 18). Thus, the minor differences seen at the tissue level between our observations and those of others are probably due to differences in the cellular composition of tissue specimens used for Western immunoblotting. Gould et al. (16) and Greenberg et al. (18) have found no expression of p36 in hepatocytes; endothelial cells within the liver, however, were found to be reactive. Using our AF5 antibody, we found the same pattern of expression by immunoperoxidase staining of normal liver tissue. p36 was found to be abundant in fibroblastic cell lines, accounting for 0.1 to 0.4% of the total protein content (25, 35). Similarly, we found that AF5 antibody reacted highly to the monkey fibroblast cell line Vero (S/N = 83). Further evidence that the protein recognized by AF5 antibody is p36 came from cloning of a cDNA library encoding AF5 antigen. AF5 antibody used as a probe recognized a fusion protein encoded by the cDNA insert that has an open reading frame encoding most of the p36 sequence (the first 306 residues of 338 amino acids). Although we have no protein sequence data, all described similarities between the

two proteins led us to believe that the hepatoma-associated AF5 antigen is indeed the protein-tyrosine kinase substrate p36.

The protein-kinase substrate p36 is also known as calpactin I heavy chain, lipocortin II, protein I, calelectrin 36, lymphocyte 33, and chromobindin 8. These proteins are structurally and functionally related to a family of phospholipid- and Ca^{2+}-binding proteins known as annexins (for recent reviews, see references 8, 20, and 23). The biologic function(s) of p36 is not known. There are, however, extensive investigations of p36 because of its potential involvement in Ca^{2+}-mediated intracellular signal transduction (3). Indeed, recent findings indicate that p36 appears to be essential for calcium-dependent exocytosis in adrenal chromaffin cells (1, 11).

As a transformation-related protein, p36 was first identified as a cellular protein substrate phosphorylated by the Rous sarcoma virus-transforming gene product $pp60^{v\text{-}src}$ (36). Subsequently, it was shown to be one of the most actively phosphorylated substrates for many of the retroviral, as well as for the epidermal growth factor and platelet-derived growth factor, receptor protein-kinases (12, 13, 23, 25, 35). Investigations indicated that p36 is also a substrate for the insulin receptor and protein kinase C (17, 26). The striking association between phosphorylation of p36 and retroviral transformation has led other investigators to believe that it may be involved in the transformation process per se (12, 23, 35). In contrast, previous studies of fibroblasts failed to show modulation of p36 expression upon cellular transformation to the malignant phenotype (12, 35). In the present investigation, we have observed a high correlation between the induction of p36 synthesis and the cellular transformation of normal hepatocytes to the malignant phenotype (HCC). This observation points towards a role for p36 in the transformation-associated events relating to this human malignancy.

Our studies demonstrated that the induced expression of p36 in HCC was not limited to cell lines derived from tumors but was also present in primary hepatic malignancy. Adult hepatocytes are resting cells but can be induced to proliferate after partial hepatectomy. In order to test whether the induction of p36 expression is due to rapid proliferation of hepatocytes, we measured transcript levels in regenerating rat liver by Northern blots. We found no expression of p36 following two-thirds hepatectomy during the first 24 h, when most of the hepatocytes have entered DNA synthesis (40). In addition, p36 was not detectable in human fetal liver at either the mRNA or the protein level. Such observations strongly suggest that induction of p36 expression in human hepatocyte transformation is not due to rapid proliferation but rather is associated with the actual malignant transformation process. Hepatocellular carcinoma cells have been shown to express epidermal growth factor and platelet-derived growth factor receptors (4, 39) that phosphorylate p36 through their protein-tyrosine kinase activities (13, 25). Further studies will be required to determine whether specific changes in p36 phosphorylation occur during transformation of normal hepatocytes to the malignant phenotype. We have observed that p36 is phosphorylated in FOCUS HCC cells under cell culture conditions (Fig. 5B) and may therefore serve as a substrate for phosphorylation in these cells. One might speculate that p36 in hepatomas is structurally and functionally similar to p36 in fibroblasts transformed by retroviral oncogenes. However, in contrast to fibroblasts, p36 is undetectable in normal hepatocytes but its synthesis is induced in the transformed state. Therefore, hepatocarcinomas offer a novel model for studying possible functions of p36 in malignant transformation.

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